Chemokines and other GPCR ligands synergize in receptor-mediated migration of monocyte-derived immature and mature dendritic cells

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Short title: Chemokines synergize in dendritic cell migration

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Key words: chemoattractants, maturation, migration, receptor expression, signal transduction, synergy

Abbreviations: BCA, bicinchoninic acid; CAM, cell-adhesion molecules; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; CI, chemotactic index; ERK, extracellular signal-regulated kinase; GAG, glycosaminoglycans; GM-CSF, granulocyte-macrophage colony stimulating factor; GPCR, G protein-coupled receptor; LPS, lipopolysaccharide; MACS, magnetic cell sorting; MAPK, mitogen-activated protein kinase; MDDC, monocyte-derived dendritic cells; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SEM, standard error of the mean

Abstract

Dendritic cells (DCs) are potent antigen presenting cells, described as the initiators of adaptive immune responses. Immature monocyte-derived DCs (MDDC) showed decreased CD14 expression, increased cell surface markers DC-SIGN and CD1a and enhanced levels of receptors for the chemokines CCL3 (CCR1/CCR5) and CXCL8 (CXCR1/CXCR2) compared with human $CD14^+$ monocytes. After further MDDC maturation by LPS, the markers CD80 and CD83 and the chemokine receptors CXCR4 and CCR7 were upregulated, whereas CCR1, CCR2 and CCR5 expression was reduced. CCL3 dose-dependently synergized with CXCL8 or CXCL12 in chemotaxis of immature MDDC. CXCL12 augmented the CCL3-induced ERK1/2 and Akt phosphorylation in immature MDDC, although the synergy between CCL3 and CXCL12 in chemotaxis of immature MDDC was dependent on the Akt signaling pathway but not on ERK1/2 phosphorylation. CCL2 also synergized with CXCL12 in immature MDDC migration. Moreover, two CXC chemokines not sharing receptors (CXCL12 and CXCL8) cooperated in immature MDDC chemotaxis, whereas two CC chemokines (CCL3 and CCL7) sharing CCR1 did not. Further, the non-chemokine G protein-coupled receptor ligands chemerin and fMLP synergized with respectively CCL7 and CCL3 in immature MDDC signaling and migration. Finally, CXCL12 and CCL3 did not cooperate, but CXCL12 synergized with CCL21 in mature MDDC chemotaxis. Thus, chemokine synergy in immature and mature MDDC migration is dose-dependently regulated by chemokines via alterations in their chemokine receptor expression pattern according to their role in immune responses.

Introduction

Chemokines are crucially involved in inflammatory responses via their capacity to recruit selective leukocyte subsets (Bonecchi et al., 2009; Raman et al., 2011). Leukocyte migration is dependent on the expression of cell-adhesion molecules (CAMs), such as selectins and integrins, which provide the driving force for leukocyte interaction with endothelial cells. Chemokine binding to extracellular matrix and chemokine receptors allow the cells to migrate along chemotactic gradients (Murphy et al., 2000; Rot and von Andrian, 2004). However, chemokine biology is more intricate than simple ligand-receptor interaction, as several studies suggest that chemokines can form dimers (Fernando et al., 2004), also after binding to glycosaminoglycans (GAG) on endothelial cells, and their receptors are found as dimers and/or oligomers at the cell surface (Johnson et al., 2005; Springael et al., 2005; Thelen et al., 2010). Moreover, multiple chemokines secreted within inflamed loci selectively enhance each other's migratory functions on leukocytes, depending on their concentration, proximity and production kinetics (Gouwy et al., 2012). Indeed, our previous findings demonstrate that at a suboptimal concentration, the CC chemokine CCL2 synergizes with the CXC chemokine CXCL8 to chemoattract monocytes (Gouwy et al., 2008). CCL3, CCL5 and CCL8, three potent mononuclear cell chemoattractants, are also able to synergize with the homeostatic chemokine CXCL12 in the migration of $CD14^+$ monocytes, $CD3^+$ T-lymphocytes or PHA-activated lymphoblasts (Gouwy et al., 2011). Similarly, homeostatic CCL19 and CCL21 were reported to enhance CCL7-induced monocyte migration (Kuscher et al., 2009).

The main function of dendritic cells (DCs) is to process and present antigens, via MHC molecules on the cell surface, to T cells of the immune system. Once activated by antigen, the immature dendritic cells in the blood or tissues migrate into lymph nodes and spleen where they act as antigen presenting cells and activate T and B cells to initiate the adaptive immune responses (Allavena et al., 2000; Banchereau et al., 2000; Sallusto and Lanzavecchia, 1999;

74 Steinman et al., 2003). DCs also play major roles in pathological processes including
75 autoimmune diseases, graft rejection and human immunodeficiency virus infection (Cameron
76 et al., 1992; Larsen et al., 1990; Steinman RM, 2003).

77 The chemotactic migratory properties of DCs in response to specific chemokines are strictly
78 regulated during their development from progenitor cells. These regulatory mechanisms are
79 implicated in mediating the trafficking of DC from blood to tissues and then to lymph nodes
80 (Dieu et al., 1998; Sato et al., 1999; Sozzani et al., 1998; Sozzani et al., 1999). Migration of
81 DC into tissues depends on a cascade of discrete events, including chemokine production and
82 regulation of chemokine receptor expression.

83 In this study, we investigated the synergy between chemoattractants in immature and mature
84 MDDC chemotaxis. In particular, we compared the synergy between CXC (i.e. CXCL8 or
85 CXCL12) and CC (i.e. CCL2, CCL3 or CCL7) chemokines in immature MDDC as well as
86 the cooperation between non-chemokine G protein-coupled receptor (GPCR) ligands (i.e.
87 fMLP or chemerin) and chemokines to chemoattract immature MDDC. Moreover, the
88 cooperation between CC (i.e. CCL3 or CCL21) and CXC (e.g. CXCL12) chemokines was
89 tested after maturation of MDDC. Due to the complexity of the chemokine binding and
90 signaling system several mechanisms have been proposed to provide an explanation for the
91 synergy between chemokines in leukocyte migration (Gouwy et al., 2012). For instance,
92 chemokine amplification of inflammatory responses could be mediated through the
93 synergistic interplay of distinct signaling pathways downstream of GPCR activation (Gouwy
94 et al., 2011). To examine the pathways involved in the synergy between CXCL12 and CCL3
95 in immature MDDC migration, cells were treated with inhibitors of signal transduction. Our
96 findings show that expression levels of chemokines and chemokine receptors regulate the
97 synergy between chemokines in chemotaxis of immature MDDC, linking the innate and
98 adaptive immune responses.

Materials and methods

Reagents

Recombinant human CCL3, CCL21 and chemerin(21-157) were obtained from R&D Systems (Minneapolis, MN). Recombinant human CXCL8(6-77) was purchased from Peprotech (Rocky Hill, NJ). The bacterial chemotactic peptide fMLP and the CXCR4 antagonist AMD3100 were from Sigma (St. Louis, MO). Synthetic intact CXCL12 and CCL7 were synthesized by solid-phase peptide synthesis using fluorenylmethoxycarbonyl (Fmoc) chemistry and were purified as described previously (Gouwy et al., 2011). Natural CCL2 was purified to homogeneity from monocyte-derived conditioned medium (Gouwy et al., 2008).

Cells

Blood was collected upon heparin, and PBMCs were seperated from granulocytes and erythrocytes by density gradient centrifugation (400*g*, 30 min, 15 °C) on Ficoll-sodium diatrizoate (Lymphoprep; Invitrogen, Groningen, The Netherlands). $CD14^+$ monocytes were isolated by MACS following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). A purity of 99% was obtained for $CD14^+$ monocytes as determined by flow cytometry. Immature MDDC were generated by incubating purified peripheral blood $CD14^+$ monocytes in plastic tissue culture dishes (10 ml/dish; 100x20 mm; 55 cm^2; International Medical products) at $1x10^6$ cells/ml in RPMI medium containing 10% FCS (Hyclone, Cramlington, UK), 50 ng/ml GM-CSF (Prospec, East Brunswick, NY) and 20 ng/ml IL-4 (Prospec) at 37°C in a CO_2 (5%) incubator for 6 days. Subsequently, for maturation, dendritic cells were incubated in fresh RPMI medium with 10% FCS containing 1 µg/ml LPS (Alexis Biochemicals, Enzo Life Science, NY, USA) for 48 h (37°C, 5% CO_2). No specific approval from an institutional review board is required for the use of buffy coats

for the following reasons: (1) no personal patient information is made available, (2) buffy coats cannot be used for treatment of patients and are therefore waste products for the blood transfusion centre and (3) blood donors sign an agreement that parts of the donation that cannot be used for patient treatment may be used for scientific research.

Flow cytometry analysis

The expression of surface molecules and chemokine receptors on $CD14^+$ monocytes, immature and mature MDDC was analysed by flow cytometry. Cells were collected and incubated for 10 min at 4°C with ice-cold FACS buffer (PBS supplemented with 2% FCS) and Fc block to prevent aspecific binding of the antibodies. For direct staining, cells ($0.3x10^6$ cells) were subsequently incubated for 30 min on ice with phycoerythrin (PE)-labeled monoclonal antibodies recognizing CCR7 (clone 150503; R&D Systems), CD80 (clone L307.4; Becton Dickinson, San Jose, CA), CD86 (clone 2331) or CD14 (clone M5E2) (BD Pharmingen, Heidelberg, Germany), fluorescein (FITC)-labeled monoclonal antibodies recognizing DC-SIGN (clone DCN46; BD Pharmingen) or CD83 (clone HB15e; Biolegend, San Diego, CA) and allophycocyanin (APC)-labeled monoclonal antibodies recognizing CD11b (clone ICRF44; BD Pharmingen) or CD1a (clone HI149; Biolegend). Cell preparations with the corresponding FITC-, PE- (BD Pharmingen) or APC-conjugated (eBioscience, San Diego, CA) isotype-matched control monoclonal antibodies were also prepared. For indirect staining, the cells were incubated with mouse anti-CXCR1 (clone 5A12), anti-CXCR2 (clone 6C6) and anti-CXCR4 monoclonal antibody (clone 12G5; BD Pharmingen), anti-CCR1 (clone 53504), anti-CCR2 (clone 48607) and anti-CCR5 (clone 45531) monoclonal antibody (R&D Systems) for 30 min on ice, washed twice with FACS buffer and subsequently stained (30 min on ice) with PE-conjugated goat anti-mouse IgG

polyclonal antibody (BD Pharmingen). Thereafter, the cells were washed twice and fixed with 4% formaldehyde in FACS buffer. Analysis of fluorescence was performed with a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest Software. The expression level of the markers and chemokine receptors between monocytes, immature and mature MDDC was compared and the corresponding p value was calculated with the Mann-Whitney (*U)* test.

Chemotaxis assay

Cell migration was assessed using a 48-well Boyden microchamber (Neuro Probe, Cabin John, MD) as described previously (Gouwy et al., 2008). In brief, immature and mature MDDC were collected from the dishes by pipetting up and down. PBS was added to each dish and the dishes were incubated for 10 min at 4 °C to detach cells. The cells were centrifuged and resuspended in chemotaxis buffer [i.e. Hanks' balanced salt solution (Invitrogen) supplemented with 1 mg/ml human serum albumin (Belgian Red Cross, Leuven, Belgium)]. Different concentrations of chemotactic factors were applied in wells of the lower compartment of the chamber and immature/mature MDDC ($1x10^6$ cells/ml) were added in the wells of the upper compartment. The lower and upper compartments were separated by a 5-µm pore-size polyvinyl pyrrolidone membrane (GE Osmonics, Minnetonka, MN). After incubation at 37 °C for 1.5 h in humidified air with 5% CO_2, the filters were removed and stained, and the cells migrated across the filter were counted microscopically (500x magnification). The results were presented as the chemotactic index (CI) calculated by dividing the number of migrated cells to the chemokine dilution through the number of cells that migrated spontaneously to the chemotaxis buffer. A chemotactic index ≥ 2 is considered to indicate that a chemotactic response to the tested chemokine was observed. Synergy

experiments were performed by adding two different chemokines together to the lower wells of the chamber. To study the effect of protein kinase inhibitors on the synergistic effect, immature MDDC were pretreated for 30 min with PD98059 (an ERK1/2 inhibitor; Merck Millipore, Overijse, Belgium) and wortmannin (a phosphatidylinositol-3-kinase inhibitor/Akt; Sigma) and loaded in the upper wells of the Boyden chamber. Statistically significant differences in CI between the combination of two chemokines and the sum of the indices obtained for the chemokines alone, as determined by the Mann-Whitney (*U)* test, are indicated by an asterisk (* $p<0.05$, **$p<0.01$).

Signal transduction assay

Immature MDDC were suspended at a concentration of $6x10^6$ cells/ml (100 µl/tube) and incubated for 30 min at room temperature in serum-free starvation medium (RPMI medium without FCS) supplemented with 0.5 g/100 ml bovine serum albumin (BSA, Sigma). After starvation, cells were stimulated at 37 °C with chemokine or a combination of chemokines (diluted in 10 µl starvation medium + 0.5% BSA) in the presence or absence of ERK1/2 or Akt inhibitors. After 5 and 30 min, signal transduction was stopped by adding ice-cold PBS. Afterwards, cells were centrifuged and cell lysis (100 µl lysis buffer/sample) was performed in PBS containing 1 mM EDTA, 0.5 % Triton X-100, 5 mM NaF, 6 M urea, protease inhibitor cocktail for mammalian tissues and phosphatase inhibitor cocktails 1 and 2 (Sigma). The lysate was incubated for 45 min on ice and clarified (10 min, 1200*g*). The protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). The amount of phosphorylated ERK1/2, Akt and p38 MAPK in the supernatant (picograms of phospho-kinase per mg of total protein) was determined using an ELISA for phospho-ERK1 (Thr202/Tyr204) plus phospho-ERK2 (Thr185/Tyr187),

phospho-Akt (S473) and phospho-p38α (T180/Y182) (R&D Systems). The ratio of phospho-ERK, phospho-Akt and phospho-p38 MAPK to total protein content was calculated for cell lysates. Then, the results are expressed relative to the phosphorylation status of ERK, Akt and p38 MAPK after control treatment. Thus, 100% corresponds to the amount of phosphorylated ERK, Akt and p38 MAPK in medium-treated cells. Statistically significant differences in kinase phosphorylation between the combination of CCL3 and CXCL12 or fMLP and the sum of the kinase phosphorylation induced by the chemoattractants alone, determined by the Sign test, are indicated by asterisks (*$p<0.05$).

Results

Expression levels of markers and chemokine receptors on $CD14^+$ monocytes, immature and mature MDDC

Immature monocyte-derived DCs (MDDC) used for migration assays were generated by culturing human peripheral blood $CD14^+$ monocytes for 6 days in medium supplemented with GM-CSF and IL-4, as evidenced (Table 1) by the significant decrease in CD14 expression (from 98.8±0.2 to 9.9±2.6% positive cells; n=6 and 15; p=0.0005) and a drastic increase in the cell surface markers DC-SIGN (from 0.1±0.1 to 88.6±3.6%; n=9 and 19; p=0.0006) and CD1a (from 0.1±0.1 to 72.2±3.5%; n=5 and 12; p=0.0007). Such immature MDDC expressed chemokine receptors with an increase for CCR1 (to 61.4±8.7% positive cells), CCR5 (to 50.2±7.2%), CXCR1 (to 33.7±13.0%) and CXCR2 (to 25.5±0.4%), but a decrease in CCR2 (to 26.3±4.7%) compared to $CD14^+$ monocytes. After a further 48 h incubation with LPS, the expression level of the DC maturation markers CD80 (83.3±9.5% positive cells; n=7; p=0.026), CD83 (60.2±9.4%; n=9; p=0.0006) and the chemokine receptors CXCR4 (19.3±4.6%; n=10; p=0.04) and CCR7 (45.0±6.9%; n=10; p=0.003) were significantly

upregulated compared to immature MDDC, whereas CCR1 (18.0±7.0%; n=9; p=0.004), CCR2 (10.1±4.0%; n=9; p=0.01) and CCR5 (20.5±4.6%; n=9; p=0.006) expression was significantly reduced on mature MDDC compared to immature MDDC (Table 1 and Fig. 1). The % CD86 positive cells was the same for the population of immature and mature MDDC, but the mean fluorescence intensity (MFI) of CD86 on mature MDDC was higher than on immature MDDC, suggesting a higher receptor density per cell (data not shown).

Synergy between CC and CXC chemokines in immature MDDC chemotaxis

Recently, we found that the CC chemokine CCL5 synergistically cooperates with the homeostatic CXC chemokine CXCL12 in monocyte migration via binding to CCR1 and CXCR4, respectively (Gouwy et al., 2011). Similarly, to assess whether the CCR1 and CCR5 ligand CCL3 was able to synergize with the CXCR4 ligand CXCL12 in immature MDDC chemotaxis, different concentrations of CCL3 and CXCL12 were added together in the lower compartment of the Boyden microchamber for chemotaxis. CCL3 alone showed a dose-response curve reaching a chemotactic index as high as 17.6±2.9 (CI±SEM) at 0.1 ng/ml, which was much higher than that of CXCL12 (Fig. 2). This finding fits with the high (≥ 50% positive cells) CCR1 and CCR5 versus low (< 10% positive cells) CXCR4 expression levels on immature MDDC (Table 1 and Fig. 1). Moreover, when CCL3 (0.03 ng/ml) was added together with different concentrations of CXCL12 (30, 100 and 300 ng/ml), the number of migrating immature MDDC was significantly increased above the sum of migrated cells obtained with the individual chemokines (Fig. 3A). In a similar experimental setting, another CXC chemokine, i.e. the CXCR1,2 agonist CXCL8 was verified for its cooperative effect with CCL3 in immature MDDC chemotaxis. CXCL8 (10 ng/ml) alone was found to significantly (p=0.02) chemoattract immature MDDC (CI±SEM of 4.7±1.8 at 10 ng/ml)

243 which is in line with the enhanced CXCR1 and CXCR2 expression (≥ 25% positive cells)
244 compared to $CD14^+$ monocytes (Fig. 3B and Table 1). Furthermore, CXCL8 (10 ng/ml)
245 significantly augmented the migration of immature MDDC to CCL3 (0.1 ng/ml) in the
246 chemotaxis assay (p=0.02) (Fig. 3B). Finally, Fig. 3C shows that synergy between the CCR2
247 ligand CCL2 (300 ng/ml) and CXCL12 (30, 100 and 300 ng/ml) to chemoattract immature
248 MDDC could only be reached at high concentrations of both ligands (Fig. 3C). A previous
249 study demonstrated that CCL2 and CXCL12 synergized to chemoattract $CD14^+$ monocytes in
250 the Boyden microchamber assay (Gouwy et al., 2008) at much lower concentrations (0.3 and
251 1 ng/ml) of CCL2 which is in line with the higher CCR2 expression levels on $CD14^+$
252 monocytes (51.6±14.2 versus 26.3±4.7% positive cells) (Table 1 and Fig. 1). These data
253 indicate the importance of chemokine receptor expression levels on the target cells which
254 correlate with the synergistic interaction capacity between chemokines in the migration of
255 leukocytes. Indeed, also lower concentrations of CXCL8 (10 ng/ml) were found to synergize
256 with CCL3 in immature MDDC migration (Fig. 3B) compared to higher concentrations of
257 CXCL8 (100 ng/ml) needed to reach synergy in $CD14^+$ monocyte migration (Gouwy et al.,
258 2008).

259 **Synergy between two CXC chemokines in immature MDDC migration**

260 As evidenced above, a combination of CC and CXC chemokines signaling via unrelated
261 receptors is providing a good chance to observe synergistic interactions. In a further attempt
262 to precisely delineate the spectrum of synergy between chemokines for immature MDDC
263 chemotaxis, the CXC chemokines CXCL8 and CXCL12 were combined on immature MDDC
264 in the Boyden microchamber assay (Fig. 4). The CXCR1/CXCR2 agonist CXCL8 and the
265 CXCR4 agonist CXCL12 alone at 30 ng/ml showed detectable immature MDDC chemotactic
266 activity, with an index of 8.1±1.4 (CI±SEM) and 2.2±0.8, respectively. Further, CXCL8 (10
267 or 30 ng/ml) significantly (p=0.037 and p=0.001, respectively) increased the chemotactic

response of immature MDDC toward 30 or 100 ng/ml CXCL12 (Fig. 4A). For example, the chemotactic index reached at 10 ng/ml of CXCL8 (6.9±1.6) and at 30 ng/ml of CXCL12 (2.2±1.0) was significantly (p=0.037) increased to 13.9±2.1 if these chemokines were combined. This is surprising since these two CXC chemokines did not synergize in monocytic cell chemotaxis (Gouwy et al., 2008). The lower expression levels of CXCR1 on monocytic cells compared to immature MDDC (5.8±5.8% versus 33.7±13% positive cells) might explain this difference. Alternatively, two CC chemokines attracting immature MDDC i.e. CCL7 (30 and 300 ng/ml) and CCL3 (0.1 and 0.3 ng/ml), sharing CCR1 as common upregulated receptor in immature MDDC, were not able to synergize in the Boyden microchamber assay when tested at various concentrations (Fig. 4B). Indeed, only a cumulative effect was observed when CCL7 and CCL3 were combined. These data indicate that chemokines competing for the same receptor do probably not synergize for chemotaxis.

Synergy between CC and CXC chemokines in mature MDDC chemotaxis

During dendritic cell maturation, the expression level of the chemokine receptors CXCR4 and CCR7 were significantly (p=0.045 and p=0.003, respectively) upregulated (from 7.5±2.4 to 19.3±4.6 and from 15.7±1.9 to 45.0±6.9 % positive cells, respectively) (Table 1 and Fig. 1). We therefore evaluated the combination of different concentrations of their ligands CXCL12 (100 and 300 ng/ml) and CCL21 (10 and 30 ng/ml) to measure mature DC migration in the Boyden chemotaxis assay (Fig. 5A). The CCR7 agonist CCL21 alone had significant chemotactic activity for mature MDDC (CI±SEM of 6.0±1.5 at 10 ng/ml), whereas CXCL12 was only weakly active (CI±SEM of 1.6±0.4 at 1 µg/ml). However, significant synergy was observed when CXCL12 (100 and 300 ng/ml) was combined with different concentrations of CCL21 (10 and 30 ng/ml) (Fig. 5A). For example, the chemotactic index reached at 10 ng/ml

of CCL21 (6.0±1.5) and at 300 ng/ml of CXCL12 (1.1±0.5) was significantly (p=0.049) increased to 18.9±2.1 if these chemokines were combined (Fig. 5A). In contrast, mature MDDC with lowered CCR1 and CCR5 expression (≤ 20% positive cells) compared to immature MDDC, did not migrate to CCL3 and CCL3 did not cooperate with CXCL12 in mature MDDC chemotaxis (Fig. 5B). The chemotactic migratory responses to inflammatory chemokines such as CCL3 were previously reported to be significantly decreased after differentiation of immature MDDC into mature MDDC (Sozzani et al., 1999).

Synergy between CC chemokines and non-chemokine GPCR-binding inflammatory mediators in immature MDDC chemotaxis and signaling

Chemerin is a chemotactic protein identified as the natural ligand of ChemR23, a G protein-coupled receptor expressed by immature MDDC (Bondue et al., 2011; Samson et al., 1998). To verify whether chemerin directly cooperates with CCL7 on immature MDDC, chemerin was added together with CCL7 in the lower compartment of the Boyden microchamber (Fig. 6A). Although chemerin is a weaker immature MDDC chemoattractant, e.g. CI±SEM of 3.5±1.5 at 1000 ng/ml, than CCL7, its chemotactic effect confirms the expression of functional chemerin receptors on immature MDDC (Samson et al., 1998; Bondue et al., 2011). Moreover, the combination of chemerin (1000 ng/ml) and CCL7 (300 ng/ml) caused a statistically significant (i.e., CI±SEM of 28.6±2.9, p=0.009) increase above the additive immature MDDC response obtained with CCL7 and chemerin alone (CI±SEM of 14.2±1.6 and 3.5±1.5, respectively). This demonstrates that the GPCR ligand chemerin and the chemokine CCL7, although binding to unrelated GPCRs, can synergize in immature MDDC chemotaxis (Fig. 6A). In addition, the combination of the non-chemokine GPCR ligand fMLP

315 (10^{-8} M) and CCL3 (0.1 ng/ml) also caused a statistically significant (p=0.049) increase above
316 the additive MDDC response obtained with fMLP and CCL3 alone (Fig. 6B).

317 Activation of GPCRs does not just lead to signaling via a single pathway, but rather triggers
318 several separate coinciding signals within the same cell. Interaction between these different
319 GPCR signal transduction pathways may result in increased cellular responses. Chemokine
320 amplification of inflammatory responses in leukocytes could be mediated through the
321 synergistic interplay of distinct signaling pathways downstream of GPCR activation (Gouwy
322 et al., 2005). To determine which signaling pathways might mediate the synergy between
323 CCL3 and fMLP in immature MDDC chemotaxis, we stimulated cells with CCL3 (0.3 or 1
324 ng/ml), fMLP (10^{-9} M) or a combination of CCL3 and fMLP for 5 min and performed ELISA
325 for phospho-ERK1/2 and phospho-Akt on the cell lysates. Figure 6C shows that immature
326 MDDC incubated for 5 min with fMLP (10^{-9} M) or CCL3 (1 ng/ml) contained higher levels of
327 phosphorylated ERK1/2 and Akt protein compared to buffer-treated cells. Furthermore,
328 synergy between fMLP and CCL3 was observed in ERK1/2 and Akt phosphorylation
329 (p=0.013 and p=0.013, respectively) in agreement with the fact that CCL3 synergizes with
330 fMLP in immature MDDC migration (Fig. 6B).

331

332 **CC and CXC chemokines cooperate in immature MDDC chemotaxis via Akt and**
333 **ERK1/2 signaling**

334 Next, we examined the involvement of the ERK1/2, Akt and p38 MAPK pathway in the
335 synergistic effect between CCL3 and CXCL12 on immature MDDC. Figure 7A shows that in
336 immature MDDC incubated for 5 min with CCL3 or CXCL12 significant phosphorylation of
337 ERK1/2 protein was induced compared with buffer-treated cells (p=0.008 and p=0.0004,
338 respectively). Furthermore, when immature MDDC were incubated for 5 min with a

combination of 0.3 ng/ml of CCL3 and 100 ng/ml of CXCL12, cooperation was observed in ERK1/2 phosphorylation compared with the sum of ERK phosphorylation by CCL3 and CXCL12 alone (p=0.02). This is in agreement with the fact that CCL3 synergizes with CXCL12 in immature MDDC migration (Fig. 3A). When the immature MDDC were incubated during 30 min with a combination of CCL3 and CXCL12, no significant enhancement in ERK phosphorylation was observed (Fig. 7A). Next, we investigated whether a combination of CCL3 (0.3 ng/ml) and CXCL12 (100 ng/ml) could enhance Akt phosphorylation in immature MDDC. CCL3 and CXCL12 induced significant Akt phosphorylation within 5 min in immature MDDC compared to buffer-treated cells (p=0.02 and p=0.005, respectively), but no synergistic effect was observed. However, the combination of CCL3 and CXCL12 cooperatively (p=0.004) induced a sustained Akt phosphorylation that could still be observed after 30 min of stimulation, a time period after which the effect of the individual chemokines was less pronounced (Fig. 7B). In contrast, no significant increase in p38 MAPK phosphorylation was detected after 5 or 30 min of stimulation of immature MDDC with CXCL12 (100 ng/ml), whereas CCL3 (1 ng/ml) induced significant (p=0.001) p38 MAPK phosphorylation within 5 min in immature MDDC compared to buffer-treated cells. Moreover, CCL3 and CXCL12 did not cooperate to enhance the phosphorylation of p38 MAPK in immature MDDC at both time points tested (Fig. 7C). Incubation of immature MDDC with the CXCR4 antagonist AMD3100 (10 μg/ml) did block the CXCL12 (100 ng/ml) induced phosphorylation of Akt after 30 min. Moreover, no cooperation was observed between CXCL12 (100 ng/ml) and CCL3 (0.1 ng/ml) in Akt phosphorylation in the presence of AMD3100 (Fig. 7D).

The effect of protein kinase inhibitors on the synergistic effect between CCL3 and CXCL12 in immature MDDC chemotaxis

To determine whether the Akt and ERK1/2 pathway mediate the synergistic effect between CCL3 and CXCL12 on immature MDDC chemotaxis, MDDC were treated with their corresponding inhibitors PD98059 or wortmannin before transfer to the upper wells of the Boyden chamber. Figure 8 illustrates that the chemotactic response to CCL3 (0.03 ng/ml) or CCL3 (0.03 ng/ml) in combination with CXCL12 (300 ng/ml) was not inhibited by PD98059. Nevertheless, a significant inhibition of the chemotactic response to CCL3 (0.03 ng/ml) and the synergy between CCL3 (0.03 ng/ml) and CXCL12 (300 ng/ml) was observed with wortmannin (100 nM). The inhibitory capacity of the PD98059 preparation was confirmed in the signaling assay. Both PD98059 (50 μM) and wortmannin (100 nM) inhibited the CCL3 (0.1 ng/ml) induced phosphorylation of ERK1/2 and Akt, respectively (data not shown). We can therefore conclude that chemotaxis of immature MDDC either induced by CCL3 as a single stimulus or in combination with CXCL12 is independent of the ERK1/2 pathway.

Discussion

Immature MDDC express a unique repertoire of inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR5, CXCR1 and CXCR2), which bind a pattern of inflammatory chemokines, including CCL2, CCL3, CCL7 and CXCL8. Dendritic cells reside in an immature state in peripheral blood or tissues where they are activated by inflammatory cytokines or antigens. After activation DC traffic via the afferent lymphatics into the draining lymph node to initiate immune responses. A dramatic change in the repertoire of chemokine receptors occurs during DC activation and maturation. Inflammatory chemokine receptors are down-regulated whereas the expression of CCR7, the receptor for CCL19 and CCL21, two

chemokines which are expressed at the luminal side of high endothelial cells and in the T cell rich areas of lymph nodes, is upregulated. This change in chemokine receptor repertoire is responsible for the migration of DC from the periphery to the draining lymph nodes (Caux et al., 2000). Simultaneously, the up-regulated cell surface receptors which act as co-receptors in T-cell activation, such as CD80 and CD83, enhance their ability to activate T cells (Lin et al., 1998; Sallusto et al., 1998; Sozzani S, 2005).

Here, we show that CXCL8 and CXCL12 significantly increase the chemotactic response of immature MDDC toward low concentrations of the CC chemokine CCL3 (Fig. 3). The chemotactic responses to the inflammatory chemokines CXCL8 and CCL3 were increased after transition of $CD14^+$ monocytes into immature MDDC, which is in line with the higher CCR1, CCR5, CXCR1 and CXCR2 expression on immature MDDC compared to $CD14^+$ monocytes (Table 1 and Fig. 1). For synergy between CXCL12 and CCL2 rather high concentrations (300 ng/ml) of the latter were required, which corresponds with reduced CCR2 expression on immature MDDC compared to monocytes (Fig. 3C and Table 1). Moreover, two CXC chemokines not sharing receptors (i.e. CXCL8 and CXCL12) also synergized in immature MDDC chemotaxis (Fig. 4A). In contrast, the combination of two CC chemokines (i.e. CCL3 and CCL7) did not provide synergy in immature MDDC chemotaxis (Fig. 4B). These latter data could implicate that chemokines competing for the same receptor (i.e. CCR1) do not synergize for chemotaxis. Interaction between CXC chemokines in plasmacytoid DC migration has been described in other studies (Krug et al., 2002; Vanbervliet et al., 2003). The cooperation between inducible CXCR3 ligands and constitutive CXCL12 may regulate recruitment of plasmacytoid DC either to lymph nodes or to peripheral sites of inflammation (Vanbervliet et al., 2003). In contrast to these plasmacytoid DC, the immature MDDC described here show only a low CXCR3 expression (7.9±1.9% positive cells; n=6).

412 Alternatively, we found that CCL3 was not active and did not cooperate with CXCL12 in
413 mature MDDC chemotaxis (Fig. 5B), whereas CCL3 synergized with CXCL12 in immature
414 MDDC chemotaxis (Fig. 3A). MDDC maturation is accompagnied by functional upregulation
415 of CCR7 and is associated with down-regulation of receptors for inflammatory chemokines
416 (e.g. CCR1/CCR5), hampering CCL3 chemotaxis and thereby explaining why CCL3 is not
417 able to cooperate with CXCL12 in mature MDDC chemotaxis. Further, it appears that most of
418 the inflammatory chemokine receptors expressed by these mature MDDC (e.g. CCR1/CCR5)
419 are not functional, whereas these receptors were operational on immature MDDC (Sallusto et
420 al., 1998). In an inflammatory environment, IL-10 causes in DC uncoupling of functional
421 receptors, which act as molecular sinks and scavengers for inflammatory chemokines
422 (D'amico et al., 2000). Indeed, DCs treated with LPS and IL-10 showed little or no migration
423 in response to CCL3, despite high expression levels of CCR1 and CCR5. Coupling of
424 chemokine receptors to chemotaxis is also regulated at the receptor signaling level (D'amico
425 et al., 2000; Sato et al., 2001). Thus, failure of CCL3 and CXCL12 to synergize in mature
426 MDDC chemotaxis can be the consequence of receptor down-regulation and/or receptor
427 uncoupling on these cells (Lin et al., 1998). Along the same line, mature MDDC express
428 CXCR4, but only marginally migrate in response to constitutive CXCL12. Earlier studies
429 have shown indeed that blood DC maturated *in vitro* apparently do not express functional
430 CXCR4 due to auto-desensitization of CXCR4 during maturation (Sallusto et al., 1998).
431 Similarly, Penna *et al.* demonstrated equal chemokine expression but distinct migration
432 patterns for blood myeloid DCs and plasmacytoid DCs (Penna et al., 2001). Moreover,
433 Humrich *et al.* described that, although CCR7 and CXCR4 were expressed at a similar level
434 on mature MDDC, CCL19 and CCL21 induced a more potent chemotactic response in mature
435 DC compared to CXCL12 (Fig. 5A) (Humrich et al., 2005). In the present study we observed
436 that at different concentrations, CXCL12 significantly elevated CCL21-induced mature

437 MDDC chemotaxis, indicating that the enhanced expression of CXCR4 on mature MDDC is
438 at least in part functional for migration. A conceivable explanation for its function on mature
439 MDDC is that CXCL12 is synergizing with CCR7 ligands to drive migration in the lymph
440 node to the site of T cell priming. Recent reports described cooperation between CCL21 and
441 CXCL12 in T lymphocyte and plasmacytoid DC migration (Bai et al., 2009; Umemoto et al.,
442 2012).

443 Furthermore, we report here that chemerin, the natural ligand of the GPCR ChemR23, was
444 able to cooperate with CCL7 in immature MDDC chemotaxis (Fig. 6A). ChemR23 is a
445 chemoattractant receptor relatively specific for antigen-presenting cells and it plays an
446 important role in the recruitment or trafficking of macrophages and dendritic cells (Samson et
447 al., 1998). The binding of chemerin to ChemR23, which is expressed on immature MDDC, is
448 important for synergy with CCL7 in chemotaxis. The non-chemokine GPCR ligand fMLP
449 also synergized with CCL3 in immature MDDC chemotaxis (Fig. 6B) and signaling (Fig. 6C).
450 Fillion et al. described a role of chemokines and formyl peptides in pneumococcal
451 pneumonia-induced monocyte/macrophage recruitment (Fillion et al., 2001). It is documented
452 that immature DCs express functional receptors (i.e. FPR, FPRL1 and FPRL2) for formylated
453 peptides (Sozzani S, 2005; Yang et al., 2000). The phenomenon of synergy between
454 chemokines and other GPCR ligands is controlled by multiple parameters, including (1) target
455 cell (different sensitivity to chemokines depending on cell type and receptor expression), (2)
456 kinetics of production, (3) dose (for each combination a different and narrow optimal *in vitro*
457 concentration range is required for each ligand, which will not necessarily reflect the optimal
458 dose *in vivo*) and (4) site of secretion (chemokines develop a gradient *in vivo* and some may
459 be quickly inactivated by NH_2-terminal cleavage depending on the presence of proteases).

460 It seems there is still much to uncover on the molecular mechanisms involved in the synergy
461 between chemokines. Experimental evidence with leukocytes has nevertheless shown that

462 after engangement of multiple chemokine receptors not only cell migration is cooperatively
463 affected but also that intracellular signaling is enhanced (Gouwy et al., 2008; Gouwy et al.,
464 2011, Mellado et al., 2001; Sebastiani et al., 2005). In this report we found that CXCL12 and
465 CCL3 cooperated in ERK1/2 and Akt phosphorylation and this synergy in signaling is
466 inhibited by the CXCR4 antagonist AMD3100 (Fig. 7D). The kinetics of synergy between
467 this CC and CXC chemokine in ERK1/2 phosphorylation was different compared to Akt
468 activation. Thus, synergy between chemokines in leukocyte migration requires the
469 coordination or cross-talk between several signal transduction cascades (Kuscher et al., 2009;
470 Mellado et al., 2001; Sebastiani et al., 2005; Vanbervliet et al., 2003). Indeed, several studies
471 showed that multiple intracellular signaling pathways are simultaneously activated by a
472 combination of chemokines. However, we report here that in all probability, the Akt pathway
473 is a major pathway mediating the synergy between CCL3 and CXCL12 in immature MDDC
474 chemotaxis (Fig. 8). Additional mechanisms for chemokine synergism, e.g. involving a single
475 chemokine receptor triggered by its agonist heterodimerized to a synergy-inducing
476 chemokine, have been proposed (Kuscher et al., 2009; Sebastiani et al., 2005). Alternatively,
477 receptor heterodimerization has been reported to explain synergism (Mellado et al., 2001).

478 In summary, our study documents the synergy between CC and CXC chemokines in
479 immature MDDC chemotaxis and provides evidence for the molecular mechanisms
480 underlying this action. Furthermore, our results show that the synergy among different
481 chemotactic receptors represent a more widespread mechanism that encompasses chemokine
482 receptors, classic chemotactic receptors (ChemR23) and formyl peptide receptors (FPR). This
483 suggests that inflammatory chemotactic receptors act in concert to promote the localization of
484 immature MDDC to inflamed peripheral non-lymphoid tissues. Antigen induced maturation
485 will subsequently cause the loss of DC responsiveness to the locally produced chemotactic

factors favoring the CCR7-dependent migration of maturing DC to secondary lymphoid organs in response to the synergistic action of CCR7 and CXCR4 ligands.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Concerted Research Actions (G.O.A.) of the Regional Government of Flanders (JVD, SS), the Fund for Scientific Research of Flanders (F.W.O.-Vlaanderen; JVD, SS), the Interuniversity Attraction Poles Program (P7/40)-Belgian Science Policy Office (JVD, SS) and AIRC (Italian Association for Cancer Research; SS).

The authors thank the members of the Blood Transfusion Center of Leuven for providing buffy coats. The technical assistance of N. Berghmans, J. Daniëls and J.-P. Lenaerts are greatly appreciated. We thank P. Proost for critical reading of the manuscript.

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Figure Legends

Figure 1: The expression of chemokine receptors on $CD14^+$ monocytes, immature and mature MDDC

FACS analysis showing chemokine receptor expression on $CD14^+$ monocytes, immature (iMDDC) and mature MDDC (mMDDC) was performed as described in Materials and Methods. One experiment representative out of 10 is shown. The shaded histograms represent the staining by the chemokine-receptor specific antibody. The open histograms represent the background staining. Numbers in the histograms indicate the mean fluorescence intensity (MFI). The shaded histograms show that positive cells for a certain chemokine receptor represent a single cell population.

Figure 2: Dose response curve of CC and CXC chemokines on immature MDDC

CCL2, CCL3, CCL7, CXCL8 and CXCL12 were evaluated for chemotactic activity on immature MDDC in the Boyden microchamber chemotaxis assay. The chemotactic activities are expressed as chemotactic indexes (CI) and the results represent the mean CI ± SEM from 4 to 7 independent experiments. †, $p<0.05$, ‡, $p<0.01$, statistically significant induced chemotaxis compared with buffer, as determined by the Mann-Whitney *U* test.

Figure 3: Synergy between CC and CXC chemokines in immature MDDC chemotaxis

Panel A and B: CCL3 (0.03 for panel A; 0.1 for panel B) was added together with CXCL12 (30 to 300 ng/ml for panel A) or CXCL8 (10 to 100 ng/ml for panel B) in the lower compartment of the Boyden microchamber to measure immature MDDC chemotaxis. **Panel**

C: CXCL12 (30 to 300 ng/ml) was combined with CCL2 (300 ng/ml) in the lower compartment of the Boyden microchamber to measure immature MDDC chemotaxis. The CI is expressed as the mean ± SEM, derived from 4 to 6 independent experiments. *$p<0.05$, **$p<0.01$, statistically significant differences in chemotactic indices between the combination of two chemokines and the sum of the indices obtained for the chemokines alone, as determined by the Mann-Whitney *U* test. †, $p<0.05$, ‡, $p<0.01$, statistically significant induced chemotaxis compared with buffer, as determined by the Mann-Whitney *U* test.

Figure 4: Synergy between two CXC chemokines in immature MDDC chemotaxis

CXCL8 (10 and 30 ng/ml) was added together with CXCL12 (30 and 100 ng/ml) (panel A) and CCL3 (0.1 and 0.3 ng/ml) was combined with CCL7 (30 and 300 ng/ml) (panel B) in the lower compartment of the microchamber to measure immature MDDC chemotaxis. The CI is expressed as the mean, derived from 5 to 7 independent experiments. *$p<0.05$, **$p<0.01$, statistically significant differences in chemotactic indices between the combination of two chemokines and the sum of the indices obtained for the chemokines alone, as determined by the Mann-Whitney *U* test. †, $p<0.05$, ‡, $p<0.01$, statistically significant induced chemotaxis compared with buffer, as determined by the Mann-Whitney *U* test.

Figure 5: Synergy between CC and CXC chemokines in mature MDDC chemotaxis

CCL21 (10 and 30 ng/ml) or CCL3 (3 and 30 ng/ml) were combined with CXCL12 (100 and 300 ng/ml) in the lower compartment of the microchamber to measure mature MDDC chemotaxis. The CI is expressed as the mean, derived from 3 to 4 independent experiments. *$p<0.05$, statistically significant differences in chemotactic indices between the combination

of two chemokines and the sum of the indices obtained for the chemokines alone, as determined by the Mann-Whitney *U* test. †, $p<0.05$, statistically significant induced chemotaxis compared with buffer, as determined by the Mann-Whitney *U* test.

Figure 6: Synergy between CC chemokines and non-chemokine GPCR-binding inflammatory mediators in immature MDDC chemotaxis and signaling

Panel A: CCL7 (30 and 300 ng/ml) was added together with chemerin (100 and 1000 ng/ml) in the lower compartment of the microchamber to measure immature MDDC chemotaxis. **Panel B:** CCL3 (0.03 and 0.1 ng/ml) was added simultaneously with the bacterial peptide fMLP (10^{-8} M and 10^{-9} M) in the lower compartment of the microchamber to measure immature MDDC chemotaxis. The CI is expressed as the mean, derived from 2 to 5 independent experiments. *$p<0.05$; **$p<0.01$ statistically significant differences in chemotactic indices between the combination of two chemoattractants and the sum of the indices obtained for the chemoattractants alone, as determined by the Mann-Whitney *U* test. †, $p<0.05$, ‡, $p<0.01$, statistically significant induced chemotaxis compared with buffer, as determined by the Mann-Whitney *U* test. Panel C: Immature MDDC were stimulated with CCL3 (1 ng/ml), fMLP (10^{-9} M) or a combination of CCL3 and fMLP. The amount of phosphorylated ERK1/2 and Akt in the cell lysates was determined by ELISA. The mean values ± SEM are derived from 7 to 8 independent experiments. 100% phosporylation corresponds to the amount of phosphorylated Akt and ERK1/2 in medium-treated cells. Statistically significant kinase phosphorylation induced by CCL3 or fMLP compared with medium-treated cells († $p<0.05$) and cooperation in signaling between CCL3 and fMLP (* $p<0.05$) was determined by the Mann-Whitney *U* test and Sign test, respectively.

Figure 7: CC and CXC chemokines cooperate in immature MDDC via Akt and ERK1/2 signaling

Immature MDDC were stimulated with CCL3 (0.3 for panel A and B; 1 ng/ml for panel C; 0.1 ng/ml for panel D), CXCL12 (100 ng/ml) or a combination of CCL3 and CXCL12 for 5 (panel A-C) or 30 min (panel A-D). In panel C immature MDDC were treated with AMD3100 (10 μg/ml) or vehicle control. The amount of phosphorylated ERK1/2, Akt and p38 MAPK in the cell lysates was determined by ELISA. The mean values ± SEM are derived from 6 to 12 independent experiments. 100% corresponds to the amount of phosphorylated Akt, ERK1/2 and p38 MAPK in medium-treated cells. Statistically significant kinase phosphorylation induced by CCL3 or CXCL12 compared with medium-treated cells († $p<0.05$, ‡ $p<0.01$) and cooperation in signaling between CCL3 and CXCL12 (* $p<0.05$, **$p<0.01$) was determined by the Mann-Whitney *U* test and Sign test, respectively.

Figure 8: Effect of protein kinase inhibitors on the synergy between CCL3 and CXCL12 in immature MDDC chemotaxis

CCL3 (0.03 ng/ml) was added together with CXCL12 (300 ng/ml) in the lower compartment of the microchamber to measure immature MDDC chemotaxis. Cells were pretreated with chemotaxis buffer (control), PD98059 (50 μM) or wortmannin (100 nM) before loading to the Boyden chamber. The CI is expressed as the mean, derived from 6 to 8 independent experiments. Statistically significant inhibition of the chemotactic response († $p<0.05$) is determined by the Sign test.

Table 1: The expression of surface markers and chemokine receptors on $CD14^+$ monocytes, immature MDDC and mature MDDC

Percentage of chemokine receptor positive cells[a]

Cell type	CCR1	CCR2	CCR5	CXCR1	CXCR2	CXCR4	CCR7
Monocytes	48.1±15.3	51.6±14.2	19.8±8.7	5.8±5.8	9.8±2.8	4.4±3.4	4.6±1.4
iMDDC	61.4±8.7	26.3±4.7	50.2±7.2	33.7±13	25.5±0.4	7.5±2.4	15.7±1.9
mMDDC	18.0±7.0	10.1±4.0	20.5±4.6	16.4±6.4	7.0±3.7	19.3±4.6	45.0±6.9

Percentage of cell surface markers positive cells[b]

Cell type	DC-SIGN	CD1a	CD11b	CD14	CD80	CD83	CD86
Monocytes	0.1±0.1	0.1±0.1	84.0±4.0	98.8±0.2	0.0±0.0	4.8±1.3	98.4±0.2
iMDDC	88.6±3.6	72.2±3.5	89.9±3.0	9.9±2.6	58.8±9.3	7.6±2.6	88.9±1.7
mMDDC	78.6±4.7	74.4±5.5	88.8±4.5	18.9±10.7	83.3±9.5	60.2±9.4	93.0±2.2

PBMCs were isolated from blood derived buffy coats. $CD14^+$ monocytes, isolated by MACS, were cultured with 50 ng/ml GM-CSF and 20 ng/ml IL-4 for 6 days to generate immature MDDC (iMDDC). For maturation (mMDDC), dendritic cells were incubated for 48 h in medium containing 1 µg/ml LPS. Expression of several chemokine receptors[a] and cell surface markers[b] was analyzed by flow cytometry with specific monoclonal antibodies. Data represent mean % positive cells ± SEM from 10 different donors.